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ANTI-MUCIN MONOCLONAL ANTIBODIES

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1. ABSTRACT

Mucins are of major interest in cell biology, not only are they highly over-expressed in many adenocarcinomas (up to 40-fold increase), but also have important physiological function, and probably more to be determined (1-3). There is much information available on mucins - doubtless because of their unusual structure being heavily glycosylated, but also containing a repeat region rich in the amino acids serine, threonine and proline. This repeat region confers high immunogenicity of the mucins, and as a result, many antibodies (Abs) have been made to mucins of different species (4). Furthermore, the production of Abs led to the cloning of the cDNAs and armed with these reagents (antibodies, cDNA and genomic

structures), advances in the knowledge of the structure and function of mucins has been rapid, together with the development of transgenic and gene knockout animals for biological studies (1-9). Here we describe monoclonal antibodies (Mabs) made to the different mucins, including Mucins 1-4, concentrating on human Mucin 1 (MUC1), to variants of MUC1, to regions outside the VNTR of MUC1, mouse Mucin1 (muc1), unusual features and cross reactions of anti-MUC1 Mabs and Abs made by patients in clinical trials. We will especially describe the Mabs produced in our laboratory.

2. INTRODUCTION

As detailed elsewhere in this volume, mucins are fascinating structures. They are large heavily glycosylated molecules which are transmembrane in nature, projecting a long way from the cell surface. However, they are not typical Type I molecules as there is cleavage and subsequent re-association of the molecules; large amounts are secreted; there is a cytoplasmic tail which signals (but to what is not as yet clear). Their function is said to be protective, anti-bacterial, lubricating, signaling, none of which appear to be altered in mice with MUC1 absent due to gene knockout by homologous recombination (9-13). However, such mice have a major defect in their immune responses. Much is known about mucins, but there is still a lot to learn about them. MUC1 can be present in great abundance - particularly in lactating breast, but also in mucinous carcinomas, particularly of the breast, ovary, and almost all types in the lung cancers (including small cell carcinoma) and in a proportion of colon cancers and adenocarcinomas from other sites (14-18). It is obvious that not all these statements fit a pattern, and for that reason, we consider that there are still more functions of mucins to be determined.

The structure of mucins have been described in detail elsewhere (1, 6), herein we will consider their basic features of relevance for antibody production (tables 1, 2). In regard to this, the molecules are heavily glycosylated and have carbohydrates that are immunogenic, available for Ab production; these are altered carbohydrates in structure and amount (sometimes more, sometimes less), thus, new epitopes on cancer cells are created. Secondly, glycosylation on the normal mucin can hide peptide epitopes, which may become exposed due to the abberant glycosylation of mucins on cancer cells. Thirdly, mucins contain a repeating region (VNTR-variable number of tandem repeats) which is highly immunogenic and indeed most Abs made to Mucins 1-4 (MUC1, MUC2, MUC3, and MUC4) essentially react with amino acids within this region. In this review, we will also describe Abs which

react with other regions, other than VNTR, of human MUC1. The immunogenic VNTR of MUC1 and of other mucins has enabled the production of a number of Abs which have been useful in histological studies, for the biochemical description of the molecules and ultimately for gene cloning using expression systems and detection by polyclonal Abs. Thus, the immunogenicity of mucins has given them a favoured position in biology. For this reason there is much information available on mucins and it is appropriate that many of the Abs be described together in this review.

This review describes Mabs to human mucins with a major focus on human MUC1 (as its description was first and knowledge of it has accumulated faster than the other mucins - this is not to indicate that it is more important than the others). In addition, a number of variants of human MUC1 are described and some of the unusual features found with Abs reacting with human MUC1, such as cross reaction with sugars occurring in mice and not humans, the unusual presentation of MUC1 peptides by MHC Class I molecules, making them the only peptides detectable by Abs to date, when bound in the MHC class I groove. In addition

MUC1 Abs have been useful for diagnostic and therapeutic studies.

3. UNIQUE FEATURES OF MUCINS FOR Mabs PRODUCTION

Mucins are of interest as they appear to be altered in both expression and structure in carcinomas. Thus far, the cDNAs of human Mucins 1-12 (MUC1-12) have been described (1, 5, 7, 19-29), particularly within the protein core region; indeed, a feature of mucins is the occurrence of a VNTR region, consisting of 24-507 repeated nucleotides - repeated up to 100 times in the case of MUC1 (1-3). Within this VNTR are multiples of serine, threonine and proline residues, which tend to form highly immunogenic hydrophilic regions with which monoclonal and polyclonal Abs react; the latter were used for the expression cloning of the mucin cDNAs. While many anti-MUC 1 Abs had previously been made by immunizing with tumor cells or crude mucin preparations eg. human milk fat globule membrane (HMFG) (17, 18, 30, 31), the cloning of the cDNAs led to the elaboration of the protein sequence and the synthesis of peptides which could be used to make further Abs (32-34).

4. MABS TO MUC1

Many Mabs to MUC1 were reported in the ISOBM TD-4 Workshop (4). Mabs can be made to different immunogens and were initially produced using breast cancer tissues, cancer cell lines or HMFG (a crude product from human milk) (table 1) (14, 17, 18, 30, 31, 35). The Mabs reacted with large heavily glycosylated molecules with molecular weight, >200Kd. The molecules were not fully described until 1988 when the cDNA sequence of MUC1 was reported and amino acid sequence deduced. With the knowledge of the amino acid sequence of MUC1, synthetic peptides were also used as antigens to produce second generation Mabs (32, 36). Longer peptides were also produced as fusion proteins (FP) (37). Currently glycosylated peptides are being synthesised for the production of Mabs; and the studies are in progress at present. In addition as the protein core is exposed in cancer, deglycosylated HMFG was prepared and used as an antigen, and Mab SM-3 was generated and it was found to be highly specific for cancer, as compared to Mabs generated to HMFG (38).

4.1. Mab TO HMFG, tumour extracts, and Muc1 VNTR peptides

4.1.1. Mab to HMFG and tumor extracts

HMFG is a crude product from human milk. The Mabs produced by immunization using HMFG include HMFG1, HMFG2, 115D8, BC2 and BC3 (table 1). These Mabs react strongly with malignant epithelial tissues, such as breast, ovary, pancreas and with MUC1 in circulation. These Mabs are not cancer specific as they also react with normal epithelial tissues, such as, found in breast, kidney, pancreas and stomach. The tissue distribution is described fully elsewhere (17, 18, 35). Some Mabs were produced using primary or metastatic breast cancer extract, such as DF3 and 3E1.2, which have similar reactivity with anti-HMFG Mabs (39-41). Most of them were demonstrated to be useful in serum test, imaging and immunochemistry studies.

Core protein of HMFG after chemical treatment to remove the peripheral oligosaccharides was also used for the production of the Mab SM3. SM3 reacted with >90% of breast cancer, but not with benign mammary tumors, normal resting or lactating breast. The characterisation of SM3 demonstrated that the epitope of SM3 appears to be masked in normal cells and exposed in malignant cells, and the glycosylation in malignant cells is aberrant.

4.1.2. Mabs to VNTR peptides

Our approach to produce Mabs was, (i) to synthesize a peptide representing the whole sequence of VNTR, with an extension into the next VNTR so that all potential amino acid epitopes were represented (table 3); (ii) to make a dimer peptide by adding a cysteine at the N-terminus (cysteine can also be added to C- or both N-, or C-termini to form a dimer or a ring form) in favour to form a native construct (32, 42-44). The synthesized peptides were conjugated to KLH using glutaraldehyde since the free peptides (<15-mer) were poorly immunogenic (45). To immunize mice the conjugated peptides were emulsified with equal volume of complete Freund's adjuvant. Mice were injected intraperitoneally and further 2 injections were given respectively after 4 and 6 weeks. Hybridomas were produced by the fusion of mouse myeloma cell line NS1, and spleen cells from immunized mice (17, 46). Four Mabs (BCP7, BCP8, BCP9, BCP10) were produced (tables 1, 3) (32). BCP8, like anti-HMFG Mabs, reacted strongly with most breast cancer and other adenocarcinomas, and less strongly with normal tissues. Like anti-HMFG Mabs BCP8 reacted with HMFG, but more strongly with deglycosylated HMFG, indicating threonine (T) in HMFG is exposed for the binding site (DTR) of BCP8, ie. amino acid T in the VNTR region is not always glycosylated (see 4.7). By contrast BCP7, BCP9 and BCP10 did not react with HMFG, but with deglycosylated HMFG, and BCP7 reacted with breast cancer, but not with normal breast indicating the anti-MUC1 peptide Mabs could be useful agent for analysing the normal and malignant mucin (32).

Producing FP is an alternative way to make a long non-glycosylated peptide, which is immunogenic and can satisfactorily be used as an antigen. A human fusion protein (hFP) containing a glutathione-S-transferase and 5 VNTR repeats of MUC1 was produced in E. coli. The 5 VNTR repeats can be cleaved from GST using the site-specific protease factor X. Two Mabs (VA1 and VA2) to MUC1 VNTR FP were produced (37, 47, 48). VA1 reacted weakly with normal breast tissues, whereas VA2 was non- reactive, but both VA1 and VA2 reacted strongly with breast and other cancers. VA1 reacted weakly with glycosylated but strongly with deglycosylated HMFG, indicating its epitope was masked in normal mucin.

4.2. Mab to non-vntr region of Muc1

Two Mabs D59 and A25 were produced to a N-terminal peptide p51-70 (tables 1, 3), which reacted with p51-70 and a fusion protein corresponding to amino acids 31-103 (including 51-70 sequence) to the N terminus of the VNTR region of MUC1. Both Mabs were non-reactive with native MUC1, such as HMFG and tissues, suggesting the epitopes are masked by carbohydrates. To date, these Abs have not been proven to be particularly useful.

4.3. Ma to cytoplasmic tail of Muc1

Two Mabs CT91 and CT1.53 were produced by immunizing rats and muc1 o/o mice using peptide CT18, which contains the last 17 amino acids of human MUC1 cytoplasmic tail, a highly conserved domain among in several species (49). These 2 Mabs reacted with the cytoplasmic peptide CT18 and also with native mucins expressed on human malignant tissues, such as breast, lung and colon cancer. Like anti-VNTR peptide Mabs, CT91 and CT1.53 reacted with normal epithelial tissues, such as, stomach and kidney, but were weak or non-reactive with normal breast. There is 88% homology (15 of 17 amino acids) in the cytoplasmic region of murine muc1 and human MUC1. CT91 and CT1.53 reacted with murine epithelial tissues, such as breast, kidney, lung, pancreas and stomach. CT91 was

successfully used in tyrosine phosphorylation studies indicating it is a useful reagent in the study of MUC1 signal transduction (11).

4.4. Mabs to a Muc1 splice variant lacking VNTR

A peptide SP11 (CYTEKNAFNSS) was made from the sequence of a unique mRNA transcript (tables 1, 3), (unpublished results). The first part of the peptide was derived from amino acids 58-62 of the MUC1 sequence (TEKNA) and second part from 343-346 (FNSS). C and Y were added at the N-terminus for potential conjugation (C) and radioactive iodination (Y). A Mab SP3.9 (IgG1) to peptide SP11 was produced which was reactive with the unique "bridging" peptide SP11 but not with other peptides from N- or C-terminus or within the VNTR of MUC1 (12). In addition, on fresh tissue sections SP3.9 was reactive with normal breast; formalin fixed tissues were non-reactive. In normal breast, staining was found only in secretions within the ducts, normal duct epithelial cells were negative. Colon, pancreas, salivary gland, stomach, kidney, liver, and lung showed no staining. In general, carcinoma tissues gave similar results, no or weak staining of cells and some staining of secretions. However, gastric carcinoma showed cytoplasmic staining, a possible variant due to the neoplastic process. Cancers of the breast, colon and lung were non-reactive as were cell lines. Thus, only native molecules in secretions were reactive, such as human milk, but no inhibition was obtained with HMFG in an inhibition ELISA assay. Some inhibition was noted with normal human serum (3/3 samples), and breast cancer serum (1/3 samples), but not with the serum of other species, such as rabbit, pig, mouse and cow. The studies indicate that the splice form is mainly expressed in secretions and could be of potential use in serum diagnostic test and in nipple discharges for diagnosis.

4.5. Mabs to secreted variants of human Muc1

Superimposed on the basic structure of mucins are different forms of MUC1, imposed by the alternate splicing of different exons (7, 50). Nucleotide sequencing of cDNA and genomic clones encoding MUC1 has revealed the existence of at least two alternative mRNA species presumed to be generated by alternative splicing of a single precursor transcript (7). Translation of the two species is expected to result in two polypeptides that share a common secretion signal sequence, N-terminus and a repeat array, but which differ in their C-terminus regions (50). The two predicted alternative forms of MUC1 are (a) one anchored at the cell surface, and (b) a second form secreted from the cell (sMUC1). The first cDNA is of traditional form which contains a hydrophobic transmembrane sequence preceding a cytoplasmic tail. The second, coding for a potential secreted form, is of interest, and contains a short stretch of 10 hydrophobic amino acids located 48 amino acids upstream of the carboxyl terminus. From its length and hydrophilicity profile (51), it was concluded, that this region probably does not act as a transmembrane domain, and that sMUC1 is likely to be secreted from the cell (7).

We have developed 3 Mabs [SEC-1 (IgG2b), SEC-2 (IgG1) and SEC-3 (IgM)] to the secreted form of MUC1 (sMUC1), the definition being by means of anti-peptide Abs made to the unique sequence (SEMVSIGLSFPMLP) which occurs only in sMUC1 (tables 1, 3). The anti-sMUC1 peptide Abs reacted with the immunizing peptide, normal and malignant tissues as well as breast cancer cell lines. They also reacted with human milk although HMFG was non-reactive, presumably due to the peptide epitope being hidden within the globule. However following deglycosylation, the HMFG became positive. Normal breast, colon and rectum tissues gave the strongest reactions, mainly staining the secretions within the glands. Cancer tissues of colon and breast were also positive and staining was observed particularly

within the inner luminal membrane of the glands. Other tissues from diseases of the bowel were also positive and the Mabs could also be of importance in other diseases than cancer. The presence of sMUC1 in secretions was examined by an inhibition ELISA wherein different components were used to inhibit the binding of the antibody to peptide. (i) The greatest inhibition was found with human milk obtained from two individuals; (ii) normal human serum also gave inhibition, as did breast cancer serum; (iii) the T47D cell lysate also gave inhibition, as did its supernatant. The breast cancer cell line MCF7 cell lysate and supernatant gave no inhibition. HMFG gave no inhibition, but after deglycosylation there was. Thus, sMUC1 is found predominantly in milk distinct from HMFG and in lesser amounts in serum and cells.

4.6. Epitope mapping of Mabs

Most epitopes of the Mabs could be determined by overlapping peptides. In our studies 6-8 overlapping peptides were synthesized on polyethylene pins to map the minimum epitope of the Mabs to the mucins (Pepscan) (Cambridge Research Biochemicals, Cambridge, UK), (43, 52) (table 1). For MUC1 Mabs twenty overlapping 6-mer peptides of MUC1 VNTR eg. PDTRPA, DTRPAP, TRPAPG, ... APDTRP were made to map the MUC1 Mabs epitopes (table 2). It should be noted that not all of the Mab epitopes could be determined by Pepscan, as there are four types of epitopes of Mabs: (i) carbohydrate; (ii) peptide; (iii) peptide and carbohydrate; and (iv) tertiary structure. Among them only type (ii) and (iii) can be determined by the Pepscan method. It was found that the epitopes of anti-MUC1 Mabs produced by HMFG were: (i) carbohydrate, such as 43 and 3E1.2, ie. the Mabs did not show any reaction with overlapping peptides nor deglycosylated HMFG; but react with H antigen or N-glycolylneuraminic acid containing carbohydrate respectively (4, 53). Other carbohydrate epitopes, such as Thomsen Friedenreich (TF), sialated lacto-N-fucopentaose III, and sial-Le^x glycolipid were also demonstrated (4). (ii) peptide, mainly within the VNTR region. A number of epitopes scattered within the VNTR region, have also been mapped for some Mabs including (BCP7, BCP9, BCP10, VA1 and VA2) which were produced by peptide and FP immunizations (tables 1, 3). This clearly indicated that regions outside the APDTR within the VNTR region are also immunogenic; (iii) peptide and carbohydrate, such as BC1, BC2 and BC3, which reacted with HMFG stronger than peptide or deglycocylated HMFG and (iv) tertiary structure, as some Mabs produced by FP or peptide could not be determined by Pepscan. This was not evident with the anti-MUC1 Mabs, but was found with MUC3 Mab M3.3 (see below) (33).

4.7. Role of glycosylation in Mab

Due to the glycosylation, mainly O-glycosylation in MUC1 molecules the Mabs reacted differently with MUC1. Some Mabs, such as 4B6 give better reaction with APDTR when the T is glycosylated ~ at least better on HMFG. Other Mabs, such as BCP8 (made to synthetic peptide ie. non-glycosylated) react better with the peptide, ie. Mabs react best with their immunogen (32, 54). An interesting finding is the substitution of the amino acids in the Mabs' epitopes with other amino acids. The Mab 4B6, produced by HMFG with epitope DTR, can have T substituted by almost every other amino acid, leads to conclude that T is not "relevant" - probably as it is glycosylated. However, BCP8, produced by immunization with peptide, with the same epitope DTR, the T can not be substituted by any amino acid. It clearly demonstrates that the amino acid T is not involved in 4B6, but it is crucial in the BCP8 epitope. Both 4B6 and BCP8 reacted with HMFG and cancer tissues, indicating that this T in the VNTR of normal and cancer mucin is not entirely glycosylated.

4.8. Muc1 APDTR is not unique to Muc1

APDTR is the dominant immunogenic region in the VNTR as most Mabs produced are reactive with APDTR. However, APDTR motif is not unique in MUC1, as there are several proteins found with this epitope. The apoptosis regulator BCL-w and Guanine nucleotide-binding protein, that contain the identical APDTR sequence found in a search for protein homology using SWISS-PROT. Molecular modelling of the 3 molecules shows the APDTR is situated on the surface, suggesting the APDTR may be involved in the function of these molecules. The APDTR amino acids are also found in rat (Guanine nucleotide-binding protein beta subunit), norcardia lactamdurans (ACV synthetase), E. coli (hydroxyperoxidase II), mycoplasma genitalium (cell division protein FTSH homolog), mycoplasma pneumoniae (cell division protein FTSH homolog), mycobacterium tuberculosis (phosphoribosylformylglycinamidine synthase II), phanerochase chrysosporium (anthranilate synthase component II), human cytomegalovirus (hypothetical protein UL30), bovine leukemia virus (hypothetical PXBL-1 protein) and saccharomyces cerevisiae (hypothetical 128.1 Kd protein). Potential cross-reactions should be taken into account when assays with MUC1 Mabs are performed.

4.9. Unusual features of Muc1 VNTR peptide binding to MHC class I molecules

We have demonstrated that peptides SAPDTRPA, SAPDTRPAP from the MUC1 VNTR, to be presented by H-2K ^{b} and HLA-A2 and the overlapping peptides RPAPGSTAP, PAPGSTAPG, APGSTAPGS to be presented by H-2D^{b} to bind with low affinity and not to contain the anchor motifs, but can induce CTL (36, 55). We have demonstrated that anti-VNTR peptide Mabs can bind to the mid and C-terminal regions of SAPDTRPAP bound to H-2K^{b} and HLA-A2 and to the mid region of APGSTAPPA bound to H-2D^{b} (51). Molecular modeling suggested that the mid (DT) and C-terminal (PAP) regions of SAPDTRPAP and the mid region of the peptide APGSTAPPA peptides looped out of the binding groove and hence accessible to anti-peptide Abs (51). In addition, the same Mabs which bind to the peptide whilst in the MHC class I groove, inhibit CTL lysis, thus such Mabs could be used to detect peptide MHC complexes in tissues as well as in cytotoxic assays. We have recently described the crystal structure of SAPDTRPA bound to MHC class I H-2K^{b} with some novel observations. [Apostolopoulos et al., submitted Immunity; also described in another chapter in this volume].

4.10. Mabs TO Muc1 for imaging and therapy

The uptake of various Abs in tumors in mice ranges from 10-50% injected dose/gm and in humans ~ 0.01%. There are numerous factors that contribute to the effective localization of Abs to tumors *in vivo* including size of Abs, epitope detected, antigen density and affinity (57). In this section we will consider MUC1 Mabs in imaging and therapy particularly the effect of their affinity.

We have previously described the affinity as being the single most important factor for the efficient localization of Abs *in vivo* (57, 58). In this study the tumor localization of a series of ^{125}I-labelled Mabs against the same antigen (Ly-2.1) with different affinities was measured as the % injected dose/gm. The maximum injected dose/gm for each Mab paralleled the magnitude of the affinity constant with greatest tumour accumulation by the Mab with the greatest affinity and the lowest tumor accumulation by the lowest affinity Mab. Furthermore, the affinity of one of the Mabs was 23 fold lower at 37° C compared to the affinity at 4° C. It is therefore important that affinities be done at 37° C, the temperature at which imaging will

be done *in vivo*. In a study comparing the localization and therapy the value of two MUC1 Mabs, BC2 and hCTMO1 were compared; it was found that rather than the absolute value of affinity the relative magnitude of the components of affinity, association and dissociation constants is important (59). The Mabs BC2 and hCTMO1 had similar affinities at 37° C of $1.36x10^7$ and $2.6x10^7$ M^{-1} but with tumor accumulation of 18% and 68% injected dose/gm respectively (Table 4) which should translate to better imaging by the hCTMO1 Mab. The major difference between the two Mabs was the association rate at 37° C of hCTMO1 ($6.04x10^3$ $M^{-1}s^{-1}$) which was ~3 times greater than that of BC2 ($2.26x10^3$ M^{-1} s^{-1}). The internalization of both Mabs to tumor cells was also studied. The hCTMO1 was vastly superior to the BC2 Mab in that 71% of the hCTMO1 was internalized while only 10-15% of BC2 was internalized. The reason was this is that when both Mabs were bound to MUC1 on the cell surface at 4° C and then warmed to 37° C, the BC2 dissociates rapidly from the cell surface and is not efficiently internalized. Both Mabs were conjugated with idarubicin and tested for *in vitro* cytotoxic activity against $MUC1^{+ve}$ tumour cells in a 24 hr [^{3}H]-thymidine uptake assay. Both conjugates were active *in vitro*, however, *in vivo* tumor inhibition of Ida-hCTMO1 was much greater than Ida-BC2 with inhibition of tumor growth by 45 and 25% respectively. Thus, for cellular antigens, particularly MUC1 the off rate (dissociation) is critical for their evaluation.

4.11. Cross reaction of MUC1 with natural Mabs

In mice, MUC1 VNTR conjugated to oxidised mannan induces $CD8^+$ CTL, high CTLp, low Abs and is accompanied with features of a T1 response with INF-gamma, IL-12 and not IL-4 or IL-10 (47, 60-62). Because of the ability to generate CTL in preclinical studies in mice, patients were immunized with MUC1 VNTR (5 repeats) conjugated to oxidised mannan in a phase I clinical study. In humans, 3/10 of patients made CTL restricted to HLA-A2, 4/15 patients generated T cell proliferative responses who were HLA-A1, DR3 and HLA-B8. A striking feature however, was the predominant Ab response where 13/25 patients made high titer Abs (63). Thus, in mice, mannan-MUC1 induced strong cellular responses whereas in humans, mannan-MUC1 generated predominantly Ab responses.

It became evident that natural anti-Gal-alpha(1,3)Gal Abs present in humans could bind to MUC1 peptide. It was therefore considered that anti-Gal-alpha(1,3)Gal Abs bound to the injected MUC1 to form immune complexes and therefore diverted the immune response to Ab rather than CTL (64, 65). We demonstrated that species which have anti-Gal-alpha(1,3)Gal Abs (humans, monkeys and chickens) developed strong Ab and weak/no CTL, and, species which expressed Gal-alpha(1,3)Gal, thus having no anti-Gal-alpha(1,3)Gal Abs (mice, MUC1 transgenic mice, rats) developed no/weak Abs and strong CTL (17, 18). In addition, Gal-alpha(1,3)Gal knockout mice, which, like humans have anti-Gal-alpha(1,3)Gal Abs, generated strong Ab responses and no CTL, whereas control, C57BL/6 mice generated the opposite response. This finding was important in that it ruled out the problem with immunization of different species, but the differences were due to the expression of Gal-alpha(1,3)Gal antigen expression and/or presence of anti-Gal-alpha(1,3)Gal Abs within each species. Furthermore, macrophages pulsed with mannan-MUC1 and injected into Gal-alpha(1,3)Gal knockout mice, now generated CTL and not Ab and this could be deviated back to strong Ab responses by incubating mannan-MUC1 and macrophages in the presence of serum containing Gal-alpha(1,3)Gal Abs (64, 65). Hence, the presence of cross-reactive Abs present in humans could explain the difference for the deviation of the immune response from predominantly cellular in mice to predominantly Ab in humans. It is clear that future clinical trials with MUC1 VNTR should be

towards *in vitro* pulsing of antigen presenting cells and this concept should be taken into account with other immunotherapeutic approaches, where cross reactive Abs may exist and deviate the immune response to an unwanted response.

4.12. Human anti-Muc1 Abs

MUC1 is not only highly immunogenic in mice in regards to Abs, but it is also immunogenic in humans. The isolation of human single-chain Fv Abs from a naive human Ab library clearly demonstrated that there are anti-MUC1 Abs to self antigen MUC1 (66). Abs were detected from B cell clones which were obtained from draining lymph nodes of patients with ovarian cancer (67, 68). Increased levels of anti-MUC1 Abs and Ab-antigen complexes were found in epithelial carcinomas which may favor the prognosis of breast cancer (69-71). However, more studies are required to confirm this.

It is of interest that patients receiving MUC1 VNTR made Abs (IgG1 and IgM isotypes) to a variety of VNTR epitopes ~ but the relevance of this to their cancer MUC1 is not clear (63). Most of the Abs reacted to the epitopes STAPPAHG and PAPGSTAP. There was no reaction with native MUC1. It is likely that cancer MUC1 is glycosylated (see above) and the Abs made reacted with immunogen, but not with the native MUC1.

Interestingly, in this study, APDTR, the predominant epitope in mice was not found to be the predominant epitope in humans immunized with mannan-MUC1. This indicates that APDTR being exposed in cancer is not immunogenic upon peptide immunization, whereas the other VNTR non-exposed (hidden) regions are highly immunogenic in humans.

5. MABS TO MOUSE MUC1

Murine and human mucin 1 molecules share a basic structure with significant protein homology: there is an extracellular region with 59-62% protein identity, a repeat region with 34% protein identity, and highly conserved transmembrane and cytoplasmic domains (87% identity in both to mouse). The cloning of the cDNAs has also led to the production of murine mucin 1 (muc1) deficient mice (o/o), which show no particular phenotype compared with normal litter mates, except for a delayed progression of transplanted mammary tumors (9). Mouse muc1 tandem repeat region consists of 16 degenerate tandem repeats with 20-21 amino acids for each repeat. Peptide Mp26 is a part of tandem repeats 2 and 3, and was synthesized (table 3). A mouse fusion protein (mFP) containing 550 bp of the mouse tandem repeat region, was produced using the bacterial expression vector pGEX-2T, into which a mouse cDNA clone, pMUC2TR, was inserted in the correct reading frame and orientation (6, 49). Mp26, mFP and CT18 were used for the immunization to produce Mabs using both muc1 o/o mice and rats. Only Mab CT1.53 against cytoplasmic tail was produced from muc1 o/o mice (see 4.3), and other 4 anti-muc1 Mabs (Mabs M30 and M70 against Mp26, and MFP25, MFP32 against mFP) were produced from rats (table 1). The 4 Mabs reacted specifically with muc1 peptides and tissues, however some cross reactivity with other mucin derived peptides was noted, particularly those containing the amino acid sequence TSS. Three different epitopes (TSS, TAVLSGTS, and LSGTSSP) of the Mabs M30, M70 and MFP25 were detected. The Mabs reacted with mucin containing murine tissues such as breast, stomach, colon, ovary, kidney, and pancreas, and the staining patterns were similar to those found in humans. Of interest was the detection of the muc1 on lymphocytes by some of the Abs - alluded to in the past (37), but not clearly described. The reactivity of these Mabs with lymphocytes in tissue sections could be seen, thus, mucins are also lymphocyte cell surface molecules as recently described in human lymphocytes and called CD227.

Indeed, mucins bear some resemblance to adhesion molecules such as sialoadhesin, (12, 13). The Mabs should prove valuable reagents when studying differentiation and expression in glandular tissues of the mouse and the ontogeny of mucin secreting tumors.

The epitopes of the anti-muc1 Mabs are entirely different from anti-human MUC1 as the homology between them is only 34% in the tandem repeat. The epitopes of anti-mouse muc1 Mabs were mapped as TSS (M30); TAVLSGTS (M70); and LSGTSSP (MFP25) using mouse muc1 overlapping peptides, derived from Mp26 (Spicer et al, 1991). The epitope for Mab MFP32 could not be determined. The sequences of the three epitopes detected were overlapped, and in particular we note that the epitopes detected by MFP25 and M30, contain the common TSS sequence.

6. MABS TO HUMAN MUC2, MUC3 AND MUC4

So far 12 different mucin genes have been cloned and most full length cDNA obtained (1, 3, 23-29). To study mucins, Mabs have been instrumental reagents in characterization, biochemical and functional experiments. However, there are some difficulties to produce purified mucins for the production of Mabs. Synthetic peptide technology has made it possible to generate Mabs to a variety of antigens, including mucins (45, 52, 72) (tables 2, 3). One drawback however, is that Mabs can be generated which only react to the immunizing peptide and not to the native molecule. In our laboratory MUC2, MUC3 and MUC4 VNTR peptides were made according their VNTR sequences (tables 2 and 4) (32-34, 73) MI29 derived from the MUC2 VNTR gene, consists of one repeat unit of 23 amino acids and part of the next repeat of four amino acids PTTT; and SIB35, derived from the MUC3 VNTR gene, containing two repeat units of 17 amino acids (33); M4.22, derived from MUC4 VNTR gene (34), corresponding to the thirty first and thirty eighth repeat (16 amino acids) and part of the next repeat (5 amino acids, TSSAS). Mabs to MUC2, MUC3 and MUC4 were produced using these synthetic peptides. Anti-MUC2 peptide Mabs CCP58, CCP31 and CCP37 were generated which reacted with both normal small and large intestine, weakly with stomach, but did not react with other normal tissues, indicating the Mabs to MUC2 are relatively specific to the gastrointestinal tract. The MUC2 Mabs also reacted strongly with colon cancer tissues and with less extent to other malignant diseases, such as breast cancer. The anti-MUC3 Mabs reacted with malignant intestine, but also with small and large intestines, and to a lesser extent with breast, lung and salivary gland tissues. There was no reaction with other normal tissues. Both anti-MUC2 and MUC3 Mabs showed the MUC2 and MUC3 are high molecular weight (>200Kd) glycoproteins (19-21, 33, 34, 73).

The cDNA of mucin 4 (MUC 4) has been cloned from human tracheo-bronchial cDNA library and the Mabs produced reacted with normal tracheo-bronchial mucosa. Two anti-MUC4 Mabs M4.171 and M4275 were produced which reacted strongly with lung cancer tissues, particularly adeno- and squamous cell carcinomas, but not with normal lung tissues by immunoperoxidase staining, indicating the glycosylation of MUC4 may be aberrant in lung cancer (protein core exposed). In addition, the Mabs reacted with normal colon. By western blotting MUC 4 was determined to have a molecular weight of 180Kd.

The epitopes of anti-MUC2, MUC3 and MUC4 Mabs were also determined by the Pepscan method. Overlapping 6-mer peptides corresponding to the VNTR regions of MUC2, MUC3, MUC 4 were also synthesized by the Pepscan method. The epitopes of Mabs to MUC2 are STTT, PTT and GTQTP for CCP31, CCP37 and CCP58 respectively. Mabs to MUC3, M3.1 and M3.3 reacted with epitopes SITTTE and PSFTSS respectively. M3.2 did not react with any of the 6-mer peptides, even though it reacted with the full length MUC3 VNTR. The

epitopes of MUC4 Mabs M4.171 and M4.275 were determined as TPL and PLPV respectively. From the mapped epitopes it clearly showed that the number of amino acids of the epitopes varied from 2 - 6amino acids. The maximum number of amino acids could be 12 amino acids as demonstrated in the MUC1 Mab workshop.

7. SERUM TESTS USING Mabs FOR CLINICAL APPLICATION

Due to up-regulation of MUC1 during cancer (up to 100 fold) it has been noted that MUC1 is shed into the serum by the cancer cells and can be detected by anti-MUC1 Mabs. A combination of the Mabs was tested and a number of serum assays were developed and are used in the clinic. Most assays showed the potential to predict prognosis, distinguish benign and malignant disease, indicate a response to therapy, or predict recurrence. Amongst all the different assays developed, CASA (cancer associated serum antigen) assay used Mabs BC2 and BC3, showed good reproducibility, sensitivity and specificity (74). CASA levels were significantly elevated in the serum of patients with breast, ovary, lung and bladder tumors, but not in those with benign conditions of these organs or pancreatic or colon cancers. When used in combination with the non-mucin CA125 assay the sensitivity and specificity could be improved. The percentage of detection of recurrence rate from 65% of patients with ovarian cancer increased to 80% when used in conjunction with CA125 (74). CA-15.3, using 2 Mabs DF3 and 115D8 in a sandwich test also showed the application in the monitoring the epithelial carcinomas (75, 76). Although great efforts have been made to develop assays using Mabs to MUC2 and MUC3 for colon cancer and MUC4 for lung cancer but there was little success.

8. PERSPECTIVE

Mabs to different mucins have been described - mouse and human mucin 1 and MUC2, MUC3 and MUC4. Most Abs are to the highly immunogenic VNTR of these mucins - they are a few to carbohydrates and some to other regions, particularly of MUC1. The Abs to mucins have been particularly useful in clearing tissue distribution on tissue sections and in the cloning of the genes - here they reacted with the repeat regions to isolate the cDNAs. Some Abs have proven useful to demonstrate useful isoforms and others *in vitro* as well as cellular signalling. Thus far anti-mucin Abs have not proven to be particular useful for serum tests for diagnosis, but for monitoring could be of value (eg CA-15.3 and CASA); or for imaging. For therapeutic value, immunoconjugates are currently being examined. Abs to mucins have played a key role in the discovery of mucins and elucidating their structure and function.

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